Molecular characterization of Newcastle disease virus (NDV) genotype VII isolated from broiler chicken flocks in Egypt

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Abstract: Newcastle disease (ND) is a highly contagious disease affects several avian species, responsible for devastating outbreaks in commercial poultry flocks not only in Egypt but also all over the world. Leading to huge annual economic losses to the national poultry industry. Despite application of various chickens' vaccination programs, the disease appears in an endemic form in commercial broiler and layer poultry farms. This study was conducted to identify the prevalent NDV strain responsible for sever outbreaks in broiler poultry farms in Middle Delta, Egypt. Twenty pathological specimens were inoculated in embryonated chicken eggs 9-11 daysvia allantioc sac revealed haemorragic dead embryos 72 hrs PI after third passage. Successful amplification of (400bp) of the fusion (F) protein by RT-PCR in 15 out of 20 tested filed isolates (75%). In addition, phylogenetic analysis based on a partial sequence of the F protein gene clustered these isolates within class II, genotype VII velogenic Newcastle disease viruses with Identity percentage of (94.2-97.7 %) with NDV-chicken /China / SDWF07l2011.
[Noura Alkhalefa, Enas K. Shawky and Abdelrazek Y. Desouky. **Molecular characterization of Newcastle disease virus (NDV) genotype VII isolated from broiler chicken flocks in Egypt.** *Life Sci J* 2017;14(5):79-86]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 11. doi:10.7537/marslsj140517.11.

Key words: NDV, RT-PCR, Fprotein, genotype VII, and phylogenetic alignment.

1. Introduction:

Poultry production is affected by many arrays of constraints, infectious diseases such as Newcastle disease, Infectious Bursal Disease, Pasteurellosis, Mycoplasmosis, and Salmonellosis, are the major ones (**Chaka et al. 2012**). Due to the sever nature and associated consequences, Newcastle disease(ND) is consider one of the major problems in chickens. The disease has become endemic in poultry population and occur every year inflicting hug losses (**Tadelle and Jobre 2004).**

NDV belongs to genus Avula virus under the family paramyxoviridae in the order Mononegavirales. The viral genome is single strand negative sense RNA and about 15 Kb in length (**De Leeuw, and Peeters**, 1999). The genome encodes usually six structural proteins in the order of 3-N–P-M-F-HN-L- 5 which are nucleocapsid protein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, haemagglutinin–neuraminidase (HN) and RNA-dependent RNA polymerase (L) protein **(Miller and Afonso., 2011)**. NDV isolates were classified into two distinct classes (class I and class II) within a single serotype (**Czegledi et al., 2006**). Each class has been classified into another different genotypes; class I (nine genotypes which ordered from 1 to 9) and class II (ten genotypes ordered from I to X). Genotype VII (class II genotype VII) was firstly categorized into two subgenotypes: VIIa, which represents viruses that emerged in the 1990s in the Far East and spread to Asia and Europe; and VIIb, which represents viruses that appeared in the Far East and spread to South Africa. Later, the two subgenotypes of VII were further classified into VIIc, d, and e, which represents isolates from China, Kazakhstan and South Africa; and VIIf, g, h, and i, which represent African isolates **(Miller et al., 2010)**. Based on the pathogenicity of NDV isolates in chickens, NDVs were divided into three main pathotypes: lentogenic strains cause mild or unapparent respiratory disease, mesogenic strains produce respiratory and nervous signs with moderate mortality and the velogenic strains cause severe intestinal lesions or neurological disease, resulting in high mortality **(Alexander, 2000)**. In Egypt, NDV outbreaks are frequently occurring, the rapid and accurate diagnosis of ND are essential. Therefore, the present study was conducted to isolate and identify the most circulating NDV strains responsible forthe recent outbreak in Middle Delta, Egypt.

2. Material and Methods

2.1. Clinical Specimens

Twenty pooled samples (proventriculus, lung, trachea, and cecal tonsils)(five from each farm) were obtained frommorbid as well as from apparently healthy broiler chicken flocks located in 2 provinces in the Middle Delta, Egypt: (Gharbia, and Kafr El sheikh) during the period from March 2015-March

2016. Most scarified chickens have diarrhea, nervous symptoms and respiratory difficulties. The specimens were grinded in phosphate buffered saline, pH 7.4, containing antibiotics of (2000 U/ml) penicillin, (2 mg/ml) streptomycin, (50 mg/ml) gentamycin and (1000 U/ml) fungizone. After three cycles of freezing and thawing, the samples were clarified by centrifugation at 5000 rpm for 10 min then the supernatants were collected and stored at - 80 °C until used.

2.2. Virus isolation in ECE

The supernatants were inoculated into 10- day-old embryonated chicken specific pathogen-free (SPF) eggs through the allantoic route using 0.2 ml/egg. The eggs were incubated at 37 °C with candling for 5 days. Eggs which show embryonic death within 24 h of inoculation were discarded and considered non-specific, whereas eggs showing embryonic death after 24 h and remaining alive up to 5 days were chilled. Allantoic fluid from each of the inoculated eggs was harvested and stored at -20c for further testing.

2.3. RNA extraction

Viral RNAs were extracted from different collected samples. Extraction was carried out according to the instruction of the RNA extraction kits QIA amp Viral RNA mini kits (Qiagen, USA). Briefly, about 140 µl from the samples was transferred to 580 µl of the Qiagen lysis buffer. Positive control extraction from freeze dried vaccine **(Himmvac La Sota Live Vaccine**) as well as Negative control extraction from the suspending transport medium will run in parallel to each sample. RNAs were eluted and stored at -80 ºC till use.

2.4. RT-PCR

The extracted RNAs were used for cDNA synthesis using Qiagen one step RT-PCR Kit (Cat. No. 210212-Qiagen, Germany) according to instruction manual. The cDNA was further used for the amplification of partial sequence of F gene of NDV through gene specific forward5' AGG AAG GAG ACA AAA ACG TTT TAT AGG3' and reverse primers5'TCA GCT GAG TTA ATG CAG GGG AGG3'. the reaction mixture consisted of 5 µl of extracted template RNA, 4.5 µl RT-PCR buffer, 1 µl of primer forward and 1 µl of primer reverse, 12.5 µl of dNTPs master mix. PCR thermo cycling using (T3 Biometra-Germany) was as follow: 20 min at 50 °C (RT reaction); 95 °C for 15 min (initial PCR activation); 39 three-step cycles of 94 °C for 30 s (denaturation), 59 °C for 40s (annealing) and 72 °C for 1 min; then 72 °C for 10 min (final extension). After amplification, 5 µl of PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide with final concentration of 0.5 µg/ml at 95 V for 30 min in 1.5 TBE buffer, against Gene Ruler TM100 bp Plus DNA Ladder (Fermentas). Images of the gels were photographed on Bio Doc Analyze Digital Systems (Biometra, Germany).

2.5. Nucleotide Sequence and phylogenetic analysis:

The RT-PCR products of four selected positive isolates were excised and purified from the gel using the QIA quick Gel Extraction Kit (Qiagen) as manufacturer instruction. The purified PCR products were sequenced using Seqscape® software for primary analysis of the raw data. A comparative analysis of sequences was performed using the CLUSTAL V multiple sequence alignment program, version 1.83 of Meg Align module of Lasergene DNA Star software **(Madison, Wisconsin, USA).** to determine nucleotide and amino acid sequence similarities and relationships. A phylogenetic tree of the nucleotide and amino acid sequences was constructed using MEGA6 software (**Tamura et al., 2013).**

3. Results:

3.1. Virus isolation:

The virus was isolated from the lung, tracheal, proventriculus, and cecaltonsils suspensions collected from the commercial broiler flocks. Congestion, stunted growth and embryonic death were observed 72hrs PI on the third egg passagecompared with non-inoculated control eggs (**Figure**. 1).

3.2. RT-PCR

Molecular identification of the virus was made by RT-PCR for the field samples as well as lyophilized vaccine (lasota strain). Out of twenty tested samples, fifteen samples were NDV positive with successfully amplification of 400 bp of fusion protein (**figure**. 2).

3.3. Sequence analysis:

In order to follow the genetic relatedness of these viral isolates, a phylogenetic tree was constructed. A group of sequences, representing some reported genotypes around the globe and previously reported from Egypt, were aligned with the sequences presented in this study using the Clustal W algorithm in Bio Edit. Multiple amino acid substitutions were observed in comparison to other NDV sequences. All sequenced samples share amino acid substitution at H57Q and I 64F, while the isolate named F198-5-NDV-F300 has amino acid substitution at T34A, F198-6-NDV-F300has amino acid substitution at Q 19 K, A 44 D and S 73 C and finally F198-7-NDV-F300has amino acid substitution at P 19 K as shown in **Figure (4)**. Studying the percent of divergence and homology between the isolated NDV and vaccinal strains showed 97.7 %) identity with NDV-chicken/China/SDWF07l2011, and (89.5-93.0 % homology to NDV-Lasota-11-fusion, NDV-isolate Hitchner fusion genes and NDV-Clone 30, respectively, and (91 %) to vectormune NDV. as shown in **Table (1)**. Phylogenetic alignment of

sequenced NDV isolates in this study with the other reference and vaccinal strains revealed that the isolated NDV strains located in a separate branch in the phylogenetic tree and it was originated from a common node and clustered more close to the Egyptian strains NDV-FU8-Egypt-NLQP-2014 and NDV-FU5-EGYPT-NLQP-2014 which revealed very virulent genotype VII **Figure (5).**

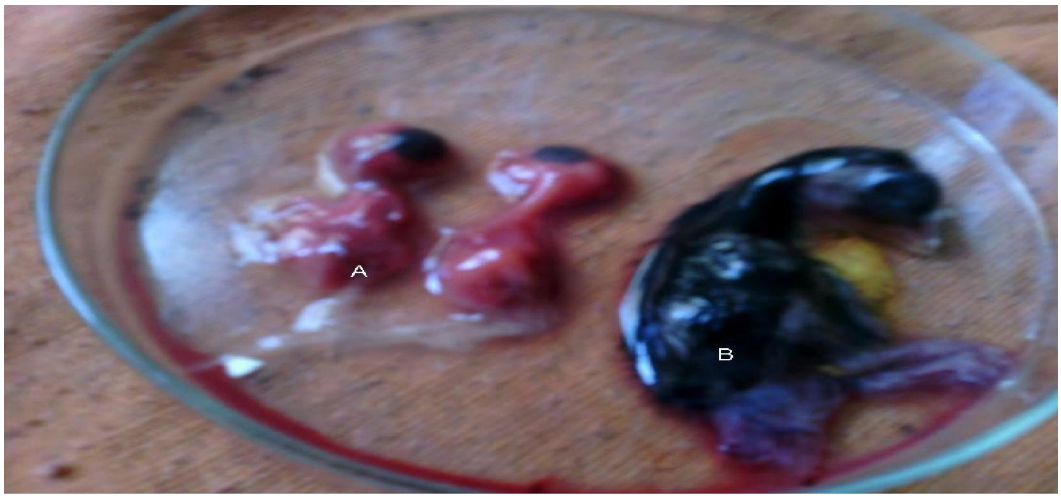

Figure (1): Embronated chicken eggs(9-11day) inoculated with the field NDV samples via allantioc sac with sever hemorrhage and embryonic death (A) compared with normal embryo(B)

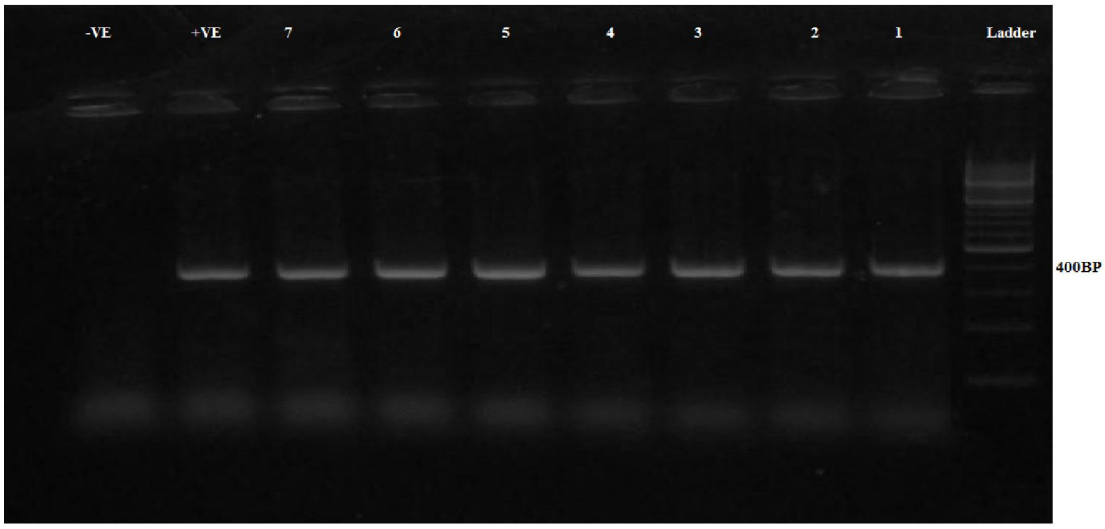

Figure (2): Agarose gel electrophoresis of the amplified PCR products of 400 bp of F gene from NDV infected field samples (Lanes 1-7). Ladder:100bp DNA ladder

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Majority
         CTTGGGGTTGCAACAGCGGCACAGATAACAGCAGCTGCGGCTCTGATACAAGCCAAACAGAA
TGCTGCCAACATCCTCCG
---------+---------+---------+---------+---------+---------+---------+---------+
10        20        30        40        50        60        70        80
---------+---------+---------+---------+---------+---------+---------+---------+
NDV-Chicken/China/SDWF07/2011
         CTTGGGGTTGCAACAGCGGCACAGATAACAGCAGCTGCGGCCCTGATACAAGCCAAACAGAA
TGCCGCCAACATCCTCCG                                                          80
NDV-LASOTA-II-fusion          .....T..C.....A.......G..C..A..T.....A.....T.....  80
NDV- isolate Hitchner fusion genes .....T..C.....A.......G..C..A..T.....A.....T.....  80
NDV-clone 30                  .....T..C.....A.......G..C..A..T.....A.....T.....  80
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NDV--VG/GA-(avenu)              .............T..C.....A..GG....G..C..A..T.................A.....T.............  80
vectormune NDV                  ..C..........C..T.................CT....T.............T..A.....T.............  80
F198-8-NDV-F300                 ..................................................C.....................  80
F198-5-NDV-F300                 ..................................................C.....................  80
F198-6-ndv-f300                 ..............................................C.....C.....................  80
F198-7-NDV-F300                 ..............................................CC.....C.....................  80

Majority
        GCTTAAAGAGAGCATTGCTGCAACCAATGAGGCTGTGCATGAGGTCACTGACGGATTATCAC
AACTATCAGTGGCAGTTG
                        ---------+---------+---------+---------+---------+---------+---------+---------+
                        90       100       110       120       130       140       150       160
                        ---------+---------+---------+---------+---------+---------+---------+---------+
NDV-Chicken/China/SDWF07/2011
        GCTTAAGGAGAGCATTGCTGCAACCAATGAAGCTGTGCATGAAGTCACCGACGGATTATCAC
AACTATCAGTGGCAGTTG       160
NDV-LASOTA-II-fusion            A.....A..........C..........G..........G.....T..........G......G.......G....  160
NDV- isolate Hitchner fusion genes A.....A..........C..........G..........G.....T..........G......G.....  160
NDV-clone 30                    A.....A..........C..........G..........G.....T..........G......G.....  160
NDV--VG/GA-(avenu)              A.....A..........C..........G..........G.....T..........G......G.....  160
vectormune NDV                  ...C..A.....................G.......C..G.....T.................G.....  160
F198-8-NDV-F300                 ......A.....................................T.....................  160
F198-5-NDV-F300                 ......A.........A...........................T.....................  160
F198-6-ndv-f300                 ......A.....................................T.C...................  160
F198-7-NDV-F300                 ......A.....................................T.....................  160

Majority
        GGAAGATGCAGCAGTTTGTTAATGACCAGTTTAATAATACGGCTCGGGAATTGGACTGCATAA
AAATTACACAGCAGGTT
                        ---------+---------+---------+---------+---------+---------+---------+---------+
                        170      180       190       200       210       220       230       240
                        ---------+---------+---------+---------+---------+---------+---------+---------+
NDV-Chicken/China/SDWF07/2011
        GGAAGATGCAGCAGTTTGTCAATGACCAGTTTAATAATACGGCGCGAGAATTGGACTGCATA
AAAATCACACAACAGGTC       240
NDV-LASOTA-II-fusion            .................T........A........A..A..T.AG.....A........C.....TG....G..A..T  240
NDV- isolate Hitchner fusion genes .................T........A........A..A..T.AG.....A........C.....TG....G..A..T  240
NDV-clone 30                    .................T........A........A..A..T.AG.....A........C.....TG....G..A..T  240
NDV--VG/GA-(avenu)              .................T........A........A..A..T.AG.....A........C.....TG....G..A..T  240
vectormune NDV                  ...........A.....T.................A..A..T.AG...........T........T.....G.....T  240
F198-8-NDV-F300                 ..........T.................A.....................................  240
F198-5-NDV-F300                 ..........T.................A.....................................  240
F198-6-ndv-f300                 ..........T.................A.........................A...........  240
F198-7-NDV-F300                 ..........T.................A.....................................  240

Majority                GGTGTAGAACTCAACCTGT
                        ---------+---------
                        250
                        ---------+---------
NDV-Chicken/China/SDWF07/2011   GGTGTAGAACTCAACCTAT
        259
NDV-LASOTA-II-fusion            ........G........G.                                                  259
NDV- isolate Hitchner fusion genes .........G........G.                                              259
NDV-clone 30                    ........G........G.                                                  259
NDV--VG/GA-(avenu)              ........G........G.                                                  259
vectormune NDV                  ..................G.                                                  259
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F198-8-NDV-F300 259
F198-5-NDV-F300 259
F198-6-ndv-f300 259
F198-7-NDV-F300 259

Figure (3): Nucleotide sequences analysis of 400bp of F gene in the NDV isolated from Middle Delta Egypt 2015 - 2016 aligned with other reference and vaccinal NDV strains. Dots indicate position where the sequence is identical to the consensus.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10		
1		95.3	95.3	95.3	93.0	94.2	97.7	96.5	94.2	96.5	1	NDV-Chicken/China/SDWF07/2011
2	4.8		100.0	100.0	97.7	96.5	93.0	91.9	89.5	91.9	2	NDV-LASOTA-II-fusion
3	4.8	0.0		100.0	97.7	96.5	93.0	91.9	89.5	91.9	3	NDV- isolate Hitchner fusion genes
4	4.8	0.0	0.0		97.7	96.5	93.0	91.9	89.5	91.9	4	NDV-clone 30
5	7.3	2.4	2.4	2.4		94.2	90.7	89.5	87.2	89.5	5	NDV--VG/GA-(avenu)
6	6.1	3.6	3.6	3.6	6.1		91.9	90.7	89.5	91.9	6	vectormune NDV
7	2.4	7.3	7.3	7.3	10.0	8.6		98.8	96.5	98.8	7	F198-8-NDV-F300
8	3.6	8.6	8.6	8.6	11.3	10.0	1.2		95.3	97.7	8	F198-5-NDV-F300
9	6.1	11.3	11.3	11.3	14.1	11.3	3.6	4.8		96.5	9	F198-6-ndv-f300
10	3.6	8.6	8.6	8.6	11.3	8.6	1.2	2.4	3.6		10	F198-7-NDV-F300
	1	2	3	4	5	6	7	8	9	10		

Table (1): Identity and diversity of the isolated NDV from Middle Delta, Egypt: (Gharbia, and Kafr El sheikh Governorate)during the period from March 2015-to March 2016. with other reference and vaccinal NDV strains. .

Majority
LGVATAAQITAAAALIQAKQNAANILRLKESIAATNEAVHEVTDGLSQLAVAVGKMQQFVNDQF
NNTAQELDCIKITQQV
---------+---------+---------+---------+---------+---------+---------+---------+
10 20 30 40 50 60 70 80
---------+---------+---------+---------+---------+---------+---------+---------+
NDV-Chicken/China/SDWF07/2011
LGVATAAQITAAAALIQAKQNAANILRLKESIAATNEAVHEVTDGLSQLSVAVGKMQQFVNDQF
NNTARELDCIKITQQV 238
NDV-LASOTA-II-fusion .....A.....K..Q.....A... 238
NDV- isolate Hitchner fusion genes .....A.....K..Q.....A... 238
NDV-clone 30 .....A.....K..Q.....A... 238
NDV--VG/GA-(avenu) .....MA.....A.....K..Q.....A... 238
vectormune NDV .....S.....N.....A.....K..Q..... 238
F198-8-NDV-F300 .....H.....I..... 238
F198-5-NDV-F300 .....T.....H.....I..... 238
F198-6-ndv-f300 .....Q.....A.....H.....I.....S..... 238
F198-7-NDV-F300 .....P.....H.....I..... 238

Majority GVELNLS

NDV-Chicken/China/SDWF07/2011 GVELNLS 259
NDV-LASOTA-II-fusion 259
NDV- isolate Hitchner fusion genes 259

NDV-clone 30	.....	259
NDV--VG/GA-(avenu)	.....	259
vectormune NDV	.....	259
F198-8-NDV-F300	.....	259
F198-5-NDV-F300	.....	259
F198-6-ndv-f300	.....	259
F198-7-NDV-F300	.....	259

Figure (4): Cluster of multiple sequence alignment of the deduced amino acid fusion protein sequence of the NDV in Middle Delta Egypt 2015-2016 in comparison to previously characterized reference and vaccinal strains.

Figure (5): Phylogenetic analysis of NDV field isolates(showed by astrixs) from Middle Delta, Egypt: (Gharbia, and Kafr El sheikh,)during the period from March 2015-March 2016. based on partial sequence of F gene with reference and vaccinal strains from gene bank, Phylogenetic tree was constructed using N-J analysis with bootstrapping (1000).

4. Discussion

Poultry play an important economic, nutritional and sociocultural role in the livelihoods in developing countries, including Egypt. The NDV is still one of the most important avian diseases of the poultry industry in Egypt, even vaccination against it for more than 60 years **(Habibian, M. et al, 2014). In Egypt** molecular epidemiology and phylogenetic analysis of NDV is an important tool to know the infection source and the possible ways of transmission that may add in NDV control **(Radwan, M.M et al, 2013).** This study was carried out to isolate genotype and assess the genetic relatedness of the isolated NDVs circulating in Middle Delta Egypt. Out of 20 tested field samples, 10 isolates

were successfully isolated on specific pathogen free embryonated chicken eggs (SPF-ECE) with severe congestion and embryonic death 72 hr PI after 3rd passages. Virus isolation technique was considered the gold standard among different techniques for virus detection, but it is time consuming and samples should be processed faster to avoid virus inactivation. which might appear negative due to loss infectivity and inappropriate sampling. Molecular identification of NDV isolates using RT-PCR for amplification of a part of F gene, revealed the presence of the amplified products at the correct expected size (400 bp) on electrophoresis in fifteen out of twenty tested samples. Our results are consistent with other studies which found that PCR is more sensitive than HA in the early detection of NDV (**Mohammed et al 2013**). NDV is a group of diverse and continuously evolving genotypes that are classified into two major classes on the basis of nucleotide sequences of the fusion (F) protein gene **(Kim et al., 2007**). Currently, NDV viruses of class II, genotypes V, VI, VII and VIII are most the predominant genotypes and are causing disease outbreaks worldwide.

NDV that are virulent for chickens have a multibasic amino acid sequence 112R/K-R-Q-K/R-R116 at the C– terminus of the F2 protein and F (phenylalanine) at residue 117, which is the N-terminus of the F1 protein, whereas the viruses of low virulence have a monobasic amino acid sequences in the same region of 112G/E-K/R-Q-G/E-R116 and L (leucine) at residue 117 (**Kim et al. 2008; Office of International Epizootes 2012).**

The genotype was confirmed by analysis of 400bp coding region of the F gene of four representative viruses (Figure 3)which showed (97 %) nucleotide homology to virulent /CH/China/SDWF07/011/V/VIId. The Phylogenetic tree was constructed using MEGA 6, showed that the Egyptian NDV isolates (F198-7-NDV-F300, F198-6-NDV-f300, F198-5-NDV-F300 and F198-8-NDV-F300) present in the same group with some of Egyptian isolates NDV-FU8-Egypt-NLQP-2014, and NDV-FU5-Egypt-NLQP-2014 which isolated in 2014 suggesting the persistence of this genotype in poultry in the country.

The presence of this genotype in Egypt may be due to be spreading in Egypt through migratory birds, as previously reported **(Mohamed et al., 2011)**. During outbreaks in poultry farms, there are some reports of wild birds becoming infected and a few mortalities in wild birds, and in countries with widespread native chicken populations. The reservoir for NDV may well be such chickens, which could be possibly infected by wild birds and become a medium for intermediary spread of infection **(Gilchrist, P. 2005)**.

Conclusions:

Many virulent NDV strains are currently circulating in the Nile Delta region of Egypt. Intensive molecular characterization is required to develop specific diagnostic tools as well as vaccine representing the local field strains.

Competing interests

The authors have declared that no competing interests exist.

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5/16/2017